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Ultrastructure of gametogenesis in the ovotestis of an estuarine pulmonate slug, *Onchidium tigrinum* (Stoliczka, 1869)

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ABSTRACT

The pulmonate slug *Onchidium tigrinum* (Stoliczka, 1869) is an estuarine protandrous gastropod. Transmission electron microscopy of both the gonadal and somatic cell populations of the ovotestis of the slug is documented. The acini of smaller slugs are comprised of developing spermatogenic cells and three to four small ill-developed oocytes. Details of the microscopic structures of Sertoli cells, interacinar cells and acinar boundary are described in-depth, revealing their secretory function. Sertoli cells are more numerous in the ovotestes of smaller slugs than in those of larger slugs. Tunnelling nanotubes of 200–400 nm in diameter are described for the first time in the Sertoli cells of molluscan ovotestis. These nanotubes may help to supply various cellular materials into distantly developing spermatogenic cells. The acini of larger slugs possess 2–3 mature oocytes along with a few spermatogenic cells. Sertoli cells, interacinar cells and spermatogonial cells are fewer in number in the acini of the ovotestis of larger individuals establishing the predominance of oogenesis in this phase of life. The number of oocytes per acinus is analysed in relation to the habitat of the pulmonates.

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Introduction

Pulmonate molluscs are hermaphrodites and the gonad is termed the ovotestis. The body size of individuals in these hermaphrodites is directly correlated to body weight and the stage of gametogenesis in the ovotestis. Pulmonates usually function as male in the early part of their sexually-active phase of life and attain simultaneous hermaphroditism in the later period of sexual life (Quattrini and Lanza 1964; Luchtel 1972a, 1972b; Tomiyama 1995; Norton et al. 2008; Koene et al. 2007; Janssen and Baur 2015). This change from male to simultaneous hermaphroditic phases is correlated with gametogenesis in the ovotestis and with alterations in other components of the reproductive system. The albumen gland has been utilised as an investigative marker of male–female role transition in hermaphroditic pulmonates—individuals with a small albumen gland are considered as male and those with a large albumen gland act as female (Cunha et al. 1998; Rakshit et al. 2014).

According to resource allocation theory (Charnov 1996), the simultaneous hermaphrodite prefers the role of sperm donor during mating because of the lower cost of sperm production relative to that of female gametes. This is consistent with the observation that during mating in hermaphrodite molluscs, individuals of larger body size/weight often assume the role of sperm recipient, while individuals of comparatively smaller body size assume the role of sperm donor (Bateman 1948; Yusa 1996; Angeloni and Bradbury 1999). It has been postulated that in simultaneous hermaphrodites size-assortative mating systems evolved to eliminate mating conflict (Angeloni et al. 2002; Angeloni 2003). It has been reported that female fecundity is significantly correlated with individuals' body size (Michiels et al. 2003; Gianguzza et al. 2005) and the reproductive success of the functional female is generally highly correlated with oocyte production (Bateman 1948). Earlier studies on the ovotestis of aquatic pulmonates such as *Biomphalaria glabrata* (Say, 1818) (De Jong-Brink et al. 1976, 1977), *Lymnaea stagnalis* (Linnaeus, 1758) (De Jong-Brink et al. 1981) and *Helisoma trivolvis* (Say, 1817) (Norton et al. 2008) had suggested that the acinus normally possesses more than one oocyte, consistent with sequential or protandrous hermaphroditism. In contrast, in terrestrial stylommatophoran pulmonates only one oocyte is produced in each acinus of the ovotestis, as reported for *Achatina fulica* (Férussac, 1821) by Rakshit et al. (2005) and *Macrochlamys indica* Benson, 1832 (Roy et al. 2016), consistent with a lesser degree of protandrous sexual development in these animals.

Cell-to-cell and tissue-to-cell communications are fundamental processes for the development and maintenance of multicellular organisms (Guo and Zheng 2004; Rustom et al. 2004; Gerdes et al. 2007; Rustom 2016). These types of communications occur at cell junctions (Bergmann et al. 1984; Hou and Maxwell

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Figure 1. Sampling area and micro-habitat of *Onchidium tigrinum*. Inset: an individual slug.

1990), including tunnelling nanotubes (TNTs) which transport various molecules and cellular organelles between spatially-distant cells (Gerdes and Rustom 2006; Davis and Sowinski 2008; Rustom 2016).

The present work investigates gametogenesis in the ovotestis of the estuarine hermaphrodite slug *Onchidium tigrinum* (Stoliczka, 1869). The study aims to establish whether there is a relationship between spermatogenesis and oogenesis and an individual's body weight. We define the comparative characteristics of gametogenesis in the ovotestes of *O. tigrinum* relative to other pulmonate molluscs.

Table 1. Comparative characteristics of ovotestes in smaller and larger *Onchidium tigrinum*.

Characteristics of ovotestis	Smaller slug	Larger slug
1. Spermatogonium	+++	–
2. Spermatocyte	+++	+
3. Spermatid	+++	++
4. Spermatozoa	+++	++
5. Periacinar cell	+	+
6. Interacinar cell	+++	+
7. Mature oocyte	–	+++
8. Follicular cell	+	+
9. Sertoli cell	+++	+
10. Sertoli–germ cell membranous connection	+++	–
11. Acinar boundary–germ cell membranous connection	+++	–

– absent; + present; ++ moderate in number; +++ high in number.

The hypothesis of this work is that the relative contributions of spermatogenesis and oogenesis within individuals are directly correlated with the body size/weight class of the slug, and that the number of oocytes per acinus are an indicator of the form of hermaphroditism.

Materials and methods

Onchidium tigrinum (Dayrat *et al*. 2011) were collected from the estuarine bank of the Hooghly River at Noorpur (22°06′N, 88°04′E), South 24-Parganas, West Bengal (Figure 1). The slugs usually hide during high tide in crevices and under debris on the estuarine mud. They emerge during low tide and browse on algae and other microorganisms. From the ecological point of view, *O. tigrinum* would not be considered to

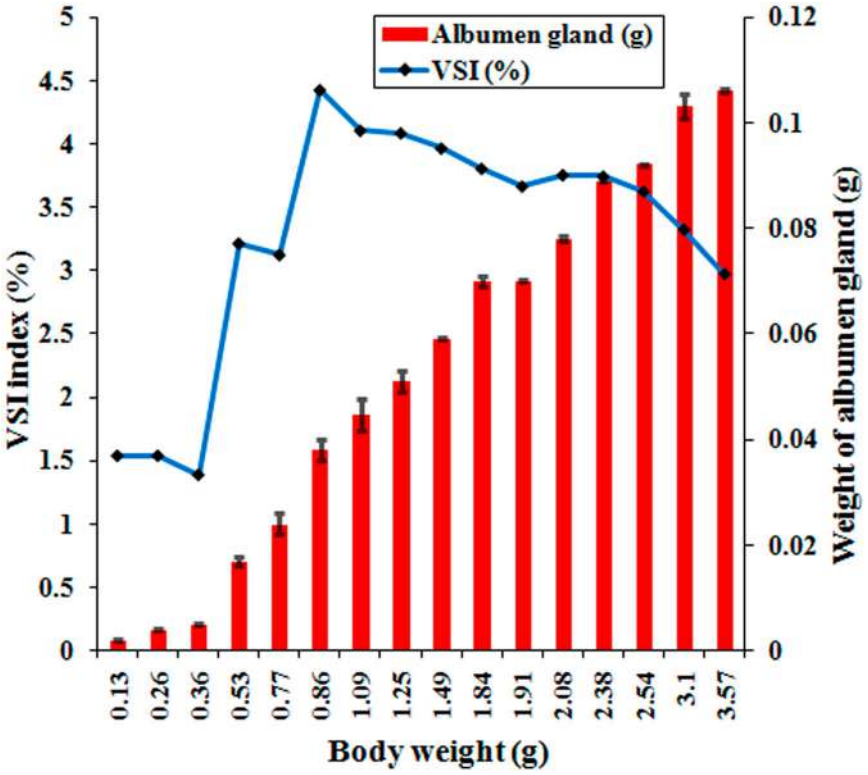

Figure 2. Histogram showing relationship between body weight and albumen gland of *Onchidium tigrinum* including vitelline somatic index (VSI). $N = 3$.

be a truly aquatic pulmonate but one at the evolutionary transition from a marine to a terrestrial, air-breathing life strategy. The animals were collected during the rainy season (June–July) when the average salinity of tidal water is c. 23.6 ppt (Sarkar et al. 2013) due to the great influx of riverine freshwater into the estuary. A group of 25 smaller slugs (0.26 ± 0.05 g body weight) with a small albumen gland and 25 larger slugs (2.38 ± 0.2 g body weight) with a large albumen gland were selected for the study. They were acclimatised for 2 days in separate cages provided with moist soil from the river area and with leafy vegetables. River water was sprayed regularly to maintain high humidity. The weight of

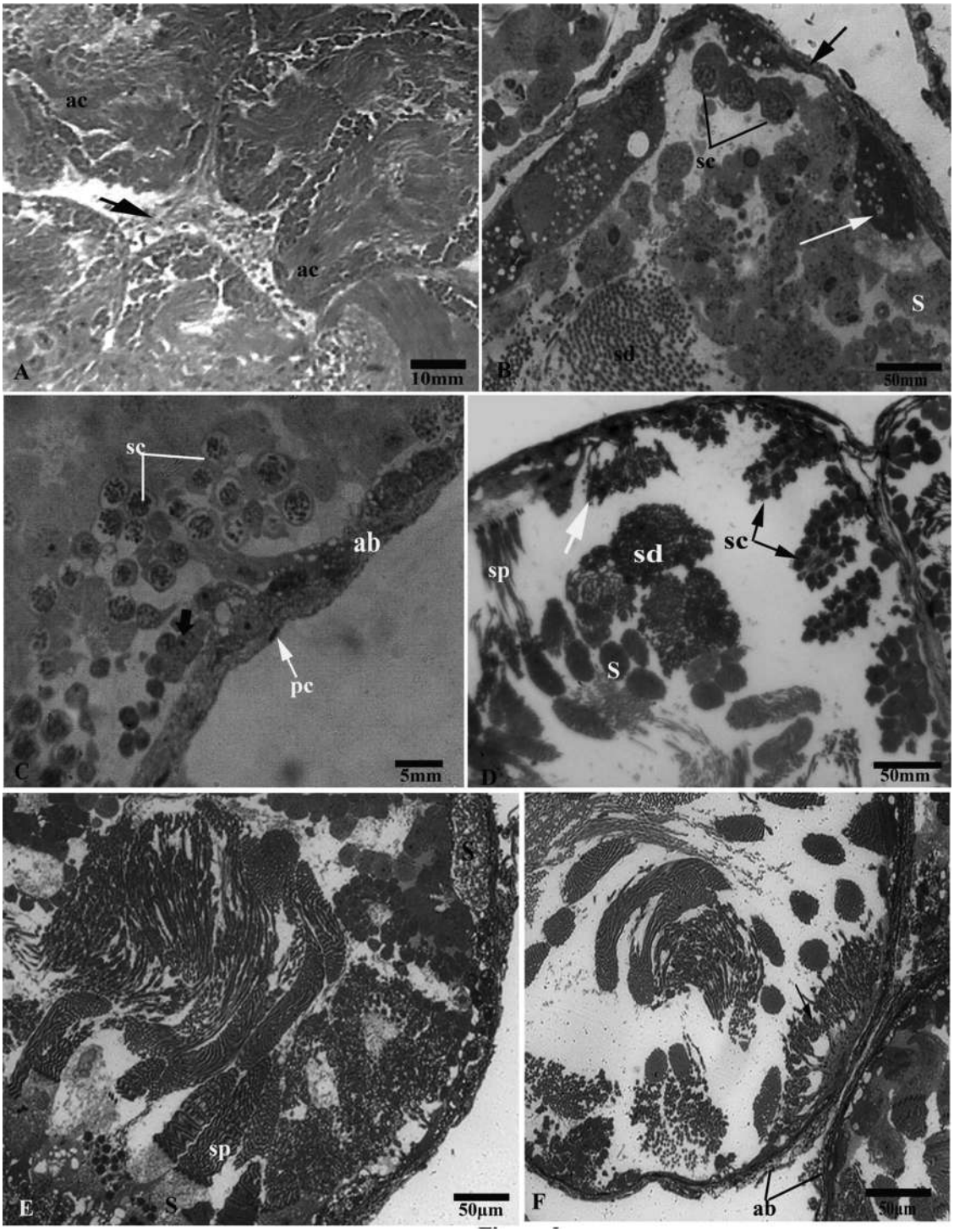

Figure 3. A–C, Light microscope photographs of the ovotestis of smaller *Onchidium tigrinum* (0.26 ± 0.05 g); A, numerous acini and aggregation of interacinar cells (arrow); B, section of acinus showing primordial oocyte (white arrow) and acinar boundary (black arrow); C, photograph showing spermatogonial cell (bold arrow), periacinar cells, and acinar boundary. **D–F,** Micrographs of semi-thin sections (1 µ); D–E, sperm bundles attached to Sertoli cells. Note the tunnelling nanotubes (TNTs) at the acinar boundary (white arrow); F, junction of acinar boundary and the TNTs (arrow). ab—acinar boundary; ac—acinus; pc—periacinar cell; S—Sertoli cell; sc—spermatocyte; sd—spermatid; sp—sperm.

the albumen glands was compared with overall body weight and a vitelline somatic index (VSI) was calculated for each slug according to the formula:

$$\text{VSI (\%)} = \frac{\text{weight of albumen gland of an individual}}{\text{body weight of the individual}} \times 100$$

The ovotestes were dissected from living slugs and immediately fixed in pre-heated (40 °C) aqueous Bouin's solution, dehydrated with ethanol and embedded in paraffin. Subsequently, 5 µm thick sections were stained with haematoxylin and eosin. Sections were observed under a light microscope.

Ovotestes were processed for ultrastructure studies using transmission electron microscopy (TEM) following the method described by Roy et al. (2016). Ten semi-thin sections of the ovotestis from five individuals of each group were studied after staining with toluidine blue (Dykstra 1993). Four copper grids, each containing five to six ultra-thin sections, were examined using a Technai Electron Microscope operated at 120 kV.

Results

The different features observed in the ovotestes of younger and older *O. tigrinum* are documented in Table 1. There was a relationship between body weight (bw) and the albumen gland of the slug. The VSI was much lower in smaller slugs (0.13–0.36 g bw) and there was a sharp increase in VSI in slugs of body weight ranging 0.36–0.86 g. A gradual decrease in VSI is found in much larger slugs of 1.09 g or more body weight (Figure 2).

The ovotestis of *O. tigrinum* was found to consist of numerous ovoid-shaped acini (Figure 3A) each containing both developing sperms and oocytes. The morphology and ultra-structural features of the ovotestis of smaller and larger slugs were broadly similar, but with some characteristic differences. Hence, the structural details of ovotestes of small and large *O. tigrinum* are documented separately below.

Smaller slugs (0.26 ± 0.005 g bw)

The cellular components of the acini were found to comprise developing stages of spermatogenic cells

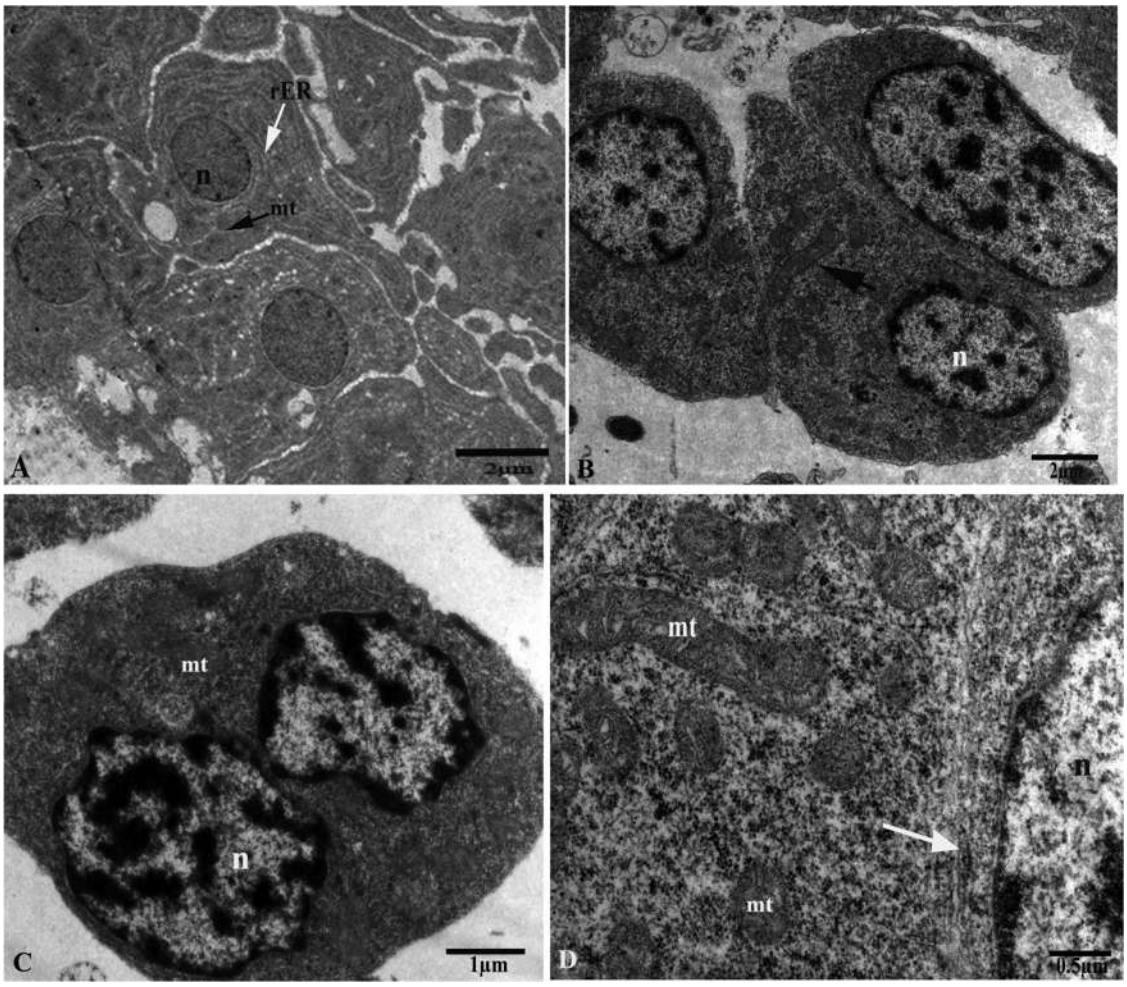

Figure 4. Transmission electron micrographs of the ovotestis of smaller *Onchidium tigrinum* (0.26 ± 0.05 g). **A,** Spermatogonia with nucleus, mitochondria and rER (black arrow); **B,** spermatocyte showing mitochondria (black arrow) and the arrangement of nuclear substances; **C,** spermatocyte displaying karyokinesis; **D,** magnified view of part of a spermatocyte showing rER (white arrow) and variety of mitochondria. mt—mitochondria; n—nucleus; rER— rough endoplasmic reticulum.

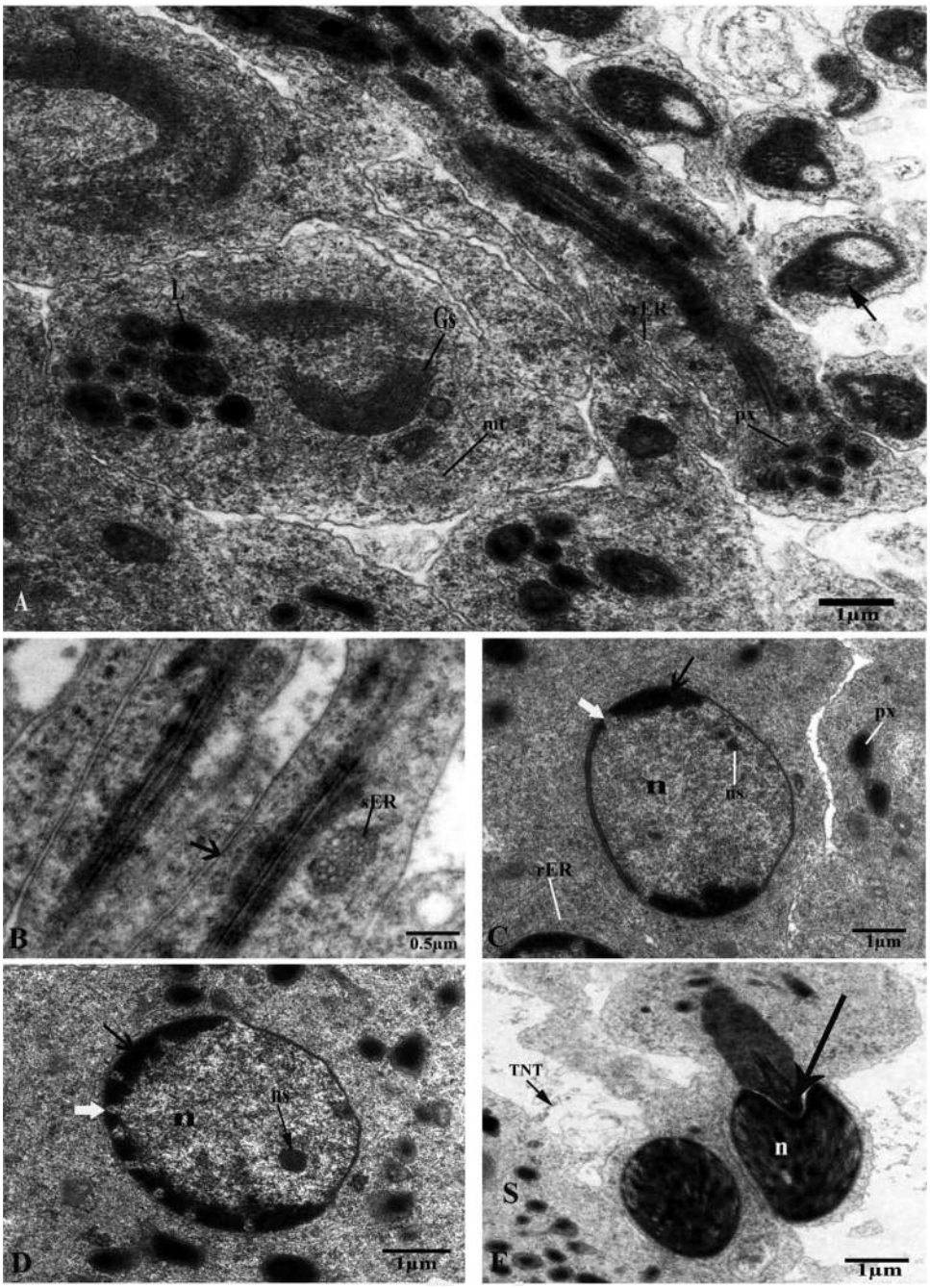

Figure 5. Transmission electron microscopy of an ovotestis acinus of smaller *Onchidium tigrinum* (0.26 ± 0.05 g). **A,** Developing spermatids and cytoplasmic components. Note the microtubular arrangement (black arrow) in the spermatid tail; **B,** longitudinal section of spermatid showing the cell junction (arrow) and the aggregation of sER in its cytoplasm; **C–D,** condensation of nuclear material (black arrow) of developing spermatid along the nuclear inner envelope, except nucleopores (white arrow); **E,** developing spermatid displaying cone shaped nucleus and budding tail (arrow). Gs—stack of Golgi apparatus; L—lysosome; mt—mitochondria; n—nucleus; ns—nucleolus; px—peroxisome; rER—rough endoplasmic reticulum; S—Sertoli cell; sER—smooth endoplasmic reticulum; TNT—tunnelling nanotube.

and three to four oocytes in the early stage of development (Figure 3B).

Spermatogenesis

Each acinus was commonly filled with four developing stages of the male gametes (spermatogonia, spermatocytes, spermatids and spermatozoa) and Sertoli cells (Figure 3C–F). The spermatogonia were mostly circular in shape with a large, compact, dense nucleus and located towards the periphery of the acini (Figure 3C, E). In many semi-thin sections these cells were observed either arranged in a chain or aggregated around Sertoli cells (Figure 3E). The spermatogonial cell cytoplasm consisted of endoplasmic reticulum (ER), mitochondria and Golgi vesicles (Figure 4A). The spermatocytes were large, with an irregular cell boundary and a nucleus containing prominent euchromatin as well as heterochromatin components (Figure 4B). Spermatocytes were distributed in the acinar lumen as aggregations of cells (Figure 3B–D). In some sections, the

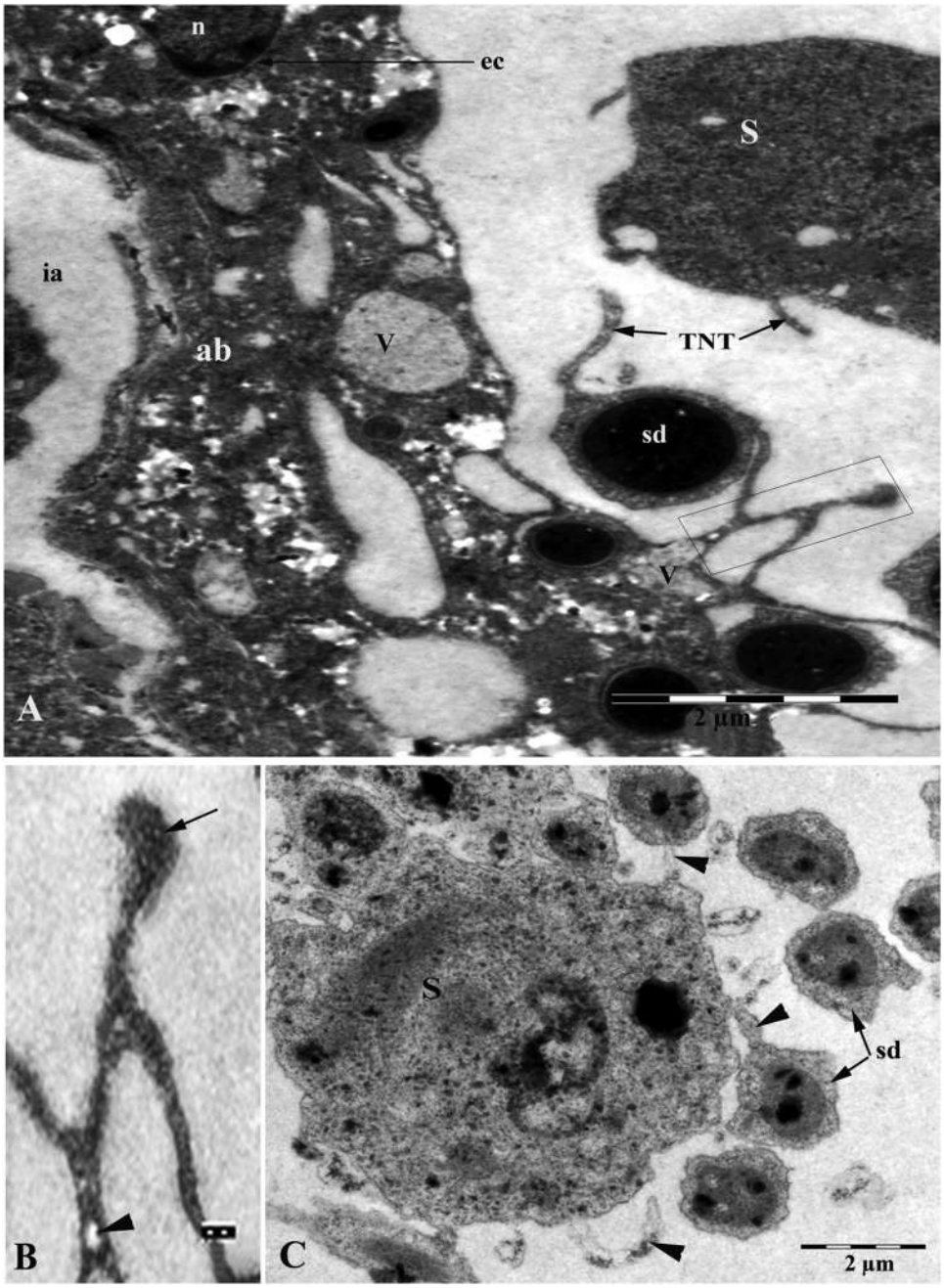

Figure 6. Ovotestis of smaller *Onchidium tigrinum* (0.26 ± 0.05 g). **A,** TNT between the developing spermatids and the cells of the acinar boundary; **B,** magnified view of a part (box) of Figure 6A showing the bulging of growing end of the TNT (arrow) and secretory droplets (arrow head). Scale bar = 500 nm; **C,** TNTs (arrow heads) between Sertoli cells and developing spermatids. ab—acinar boundary; ec—elongated nucleated cell; ia—interacinar space; n—nucleus; S—Sertoli cell; sd—spermatid; V—secretory vesicle.

spermatocytes exhibited karyokinesis (Figure 4C). Cytoplasmic contents comprised rough endoplasmic reticulum (rER), mitochondria and the Golgi apparatus (Figure 4B, D). The spermatids were observed to be distributed throughout the acini (Figure 3B, D–E). In most sections spermatids were found aggregated in bundles of cells and exhibiting different stages of development, including one form with a manchettee (Figures 3D–E, 5A). The cytoplasm of developing spermatids contained the Golgi apparatus, lysosomes, rER, smooth endoplasmic reticulum (sER) and peroxisomes (Figure 5A–C). The tail part of these cells exhibited a typical 9 + 2 microtubular arrangement within a membranous sheath. The nuclear materials of developing spermatids were packed throughout the inner membrane of the nuclei but with gaps, here termed nuclear pores (Figure 5C). As a result, this nuclear condensation exhibited a specific pattern with gaps of various sizes (Figure 5D). Finally the spermatids attain a cone-shaped nucleus in the head region (Figure 5E) that characteristically becomes more elongated to the form seen in spermatozoa. The formation of the tail bud in spermatids developed at the nuclear fossa posterior to the nucleus (Figure 5E). The developing spermatogenic cells were found scattered throughout the acinar space and communicating with the cells of the acinar

boundary and the Sertoli cells via thin membranous channels similar to TNTs (Figure 6A–C). Small dense granules and secretory droplets were observed within cytoplasmic channels (Figure 6B). The free ends of these channels were slightly swollen and contained dense secretory materials (Figure 6A–B). These channels were comparatively longer and more commonly found in the acini of smaller slugs than in those of larger. The spermatozoa within the ovotestis acini were aggregated, with their pointed nuclear heads inserted into the Sertoli cell (Figure 3D–E) and the tails free within the acinar lumen (Figure 3D).

Sertoli cells

The Sertoli cells are large elongated columnar cells located on the inner boundary of the acinar wall (Figure 7A–B). The cell cytoplasm consisted of rER, dense secretory droplets, membrane-bound vesicles and Golgi apparatuses and associated secretory granules (Figure 7B–F). The free ends of these cells were oriented

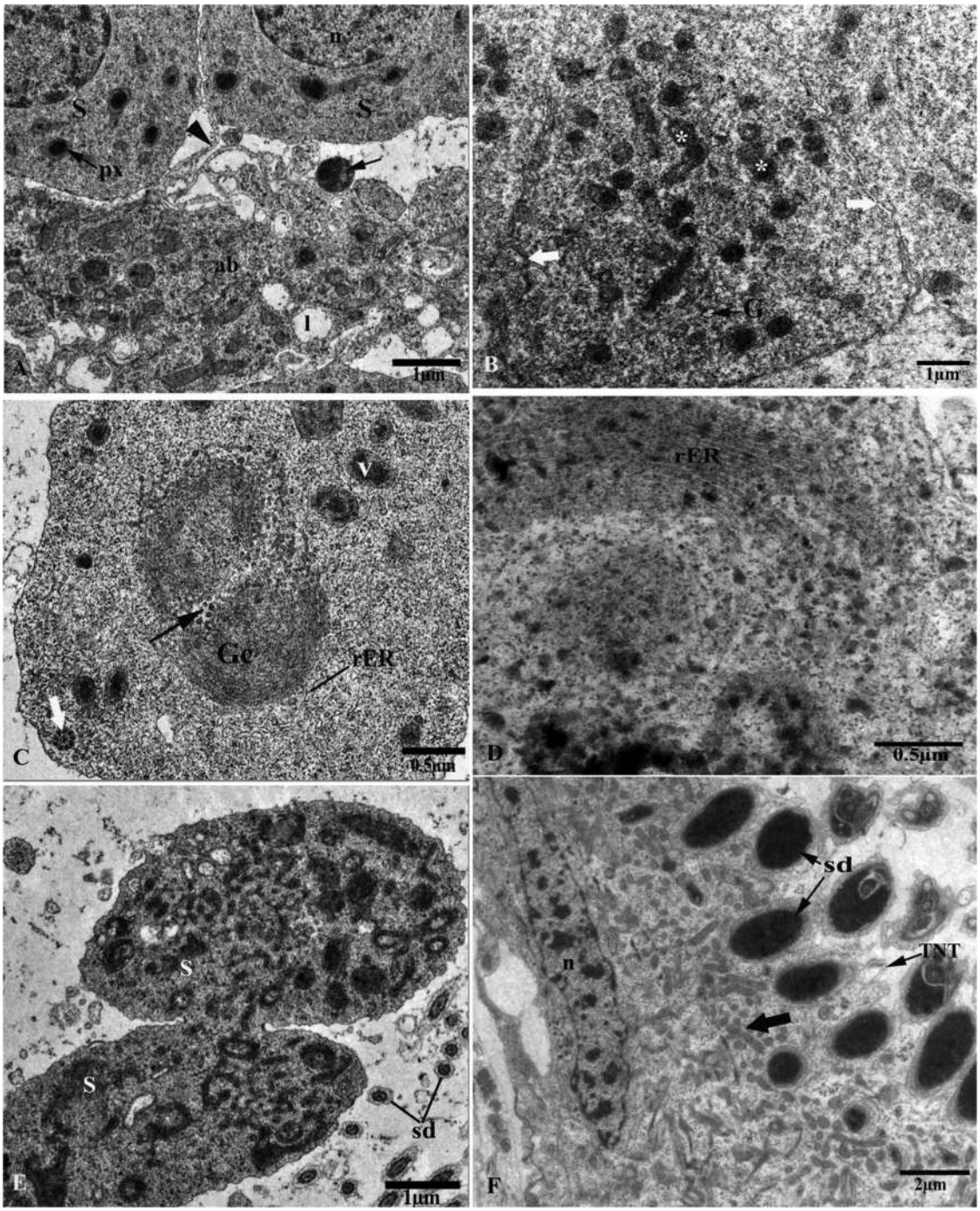

Figure 7. Ovotestis of smaller *Onchidium tigrinum* (0.26 ± 0.05 g). **A,** Connections between acinar boundary and Sertoli cells (black arrow). Note the secretory proteins (arrow head) in the acinar boundary; **B,** Sertoli–Sertoli junctions (white arrows) and secretory proteins (*); **C,** cytoplasm of Sertoli cell showing secretory granules (arrow), rER, spermatid tail (white arrow) and secretory vesicle; **D,** rER in the cytoplasm of Sertoli cell; **E,** dividing stage of a Sertoli cell; **F,** developing spermatids in the cytoplasm of Sertoli cell; bold arrow indicates secretory vesicles of different sizes. ab—acinar boundary; Gc—Golgi centre; l—lipid droplet; n—nucleus; px—peroxisome; rER—rough endoplasmic reticulum; sd—spermatid; S—Sertoli cell; TNT—tunnelling nanotube; v—vesicle.

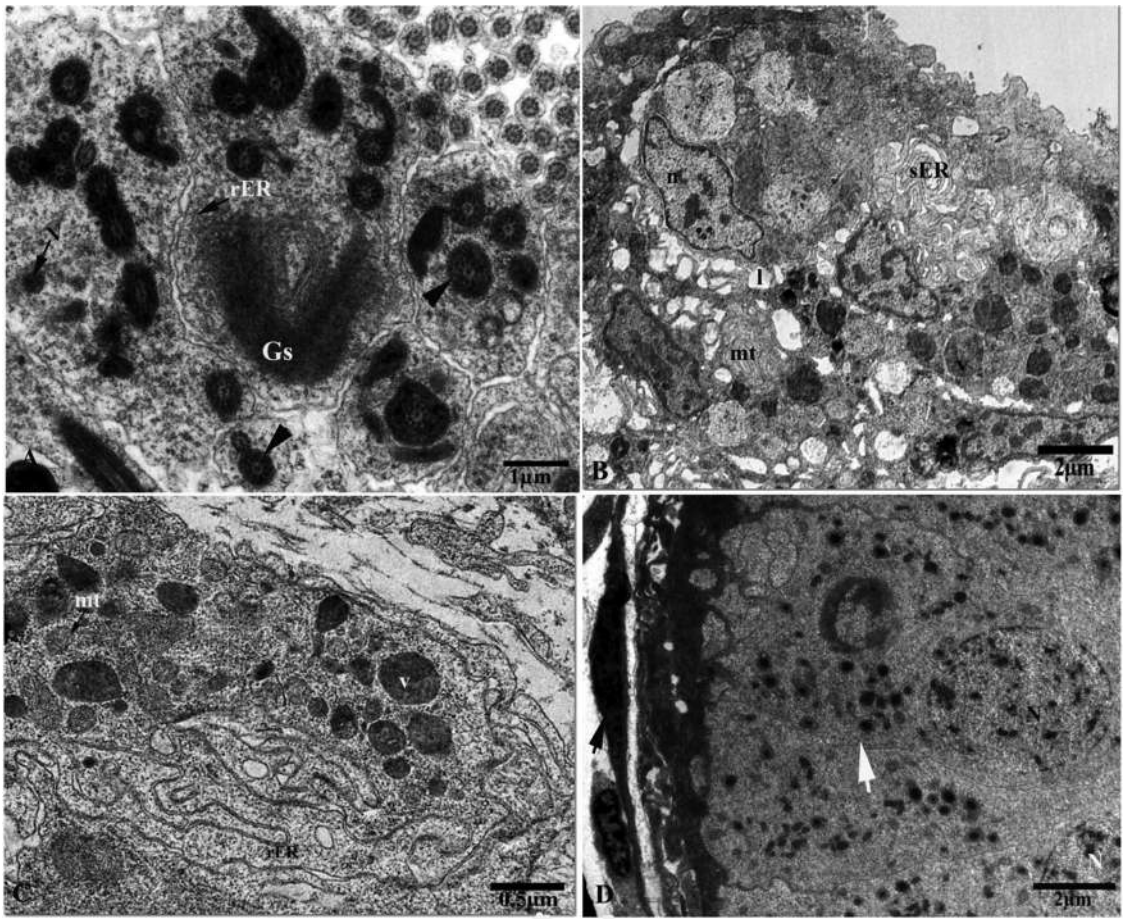

Figure 8. Ovotestis of smaller *Onchidium tigrinum* (0.26 ± 0.05 g). **A,** Sertoli cells displaying spermatids tail (arrow head) and cytoplasmic components; **B,** aggregation of interacinar cells displaying secretory vesicles and sER; **C,** magnified view of an interacinar cell cytoplasm and its components; **D,** periacinar cells (black arrow) around the acinar boundary. Note the thin round nucleus in functional Sertoli cells and Sertoli–Sertoli cell junction (white arrow). Gs—Golgi stack; l—lipid droplets; mt—mitochondria; n—round nucleus; rER—rough endoplasmic reticulum; sER—smooth endoplasmic reticulum; v—vesicle.

towards the acinar lumen and possessed cytoplasmic tunnels of 200–400 nm diameters (Figures 3E–F, 7F). The nanotubules carried some cytoplasmic granules and connected the developing spermatogenic cells to the Sertoli cells (Figure 3F). The Sertoli–Sertoli cell junction bore very short membranous digitations (Figure 7B). In most TEM sections it was found that the different developing stages of spermatogenic cells adhered tightly to the cytoplasm of Sertoli cells (Figures 7F, 8A). The nuclei of the cells were large, but their shape varied depending on the functional state of the cells (Figure 7A). The cells interpreted as being in the more active state possessed small round nuclei (Figure 7A) containing few euchromatin bodies. Some TEM sections showed the dividing Sertoli cells with prominent cytoplasmic connections (Figure 7E).

Interacinar components and the acinar boundary

The interacinar space contained connective tissues, short interacinar cells and slightly elongated periacinar cells (Figure 3A, C). The interacinar cells were oval in shape and distributed in the regions between acini, either singly or in aggregations (Figures 3A, 8B). The nucleus of these cells was large, but irregular in shape. The cytoplasm of the cell was observed to be composed of rER, sER, lipid droplets, secretory vesicles, mitochondria and Golgi apparatus (Figure 8B–C). The periacinar cells were flattened and occupied largely by the nucleus (Figures 8D, 9A). The cells formed a thin layer with smooth muscles around each acinus. Muscle cells were observed distributed along the outer boundary of each acinus; they probably promote the movement of developing spermatozoa through the acinar lumen. The junction between the periacinar cell and the acinar boundary was filled with connective tissues and muscle fibres.

The acinar boundary was found to be a membranous structure that surrounds each acinus (Figures 3C–D, 8D, 9B). This boundary was composed of elongated dense nucleated cells, containing mitochondria and small secretory granules as well as secretory droplets, lipid droplets and some fibrous structures (Figure 9B–C). Cytoplasmic channels were also present, extending from the acinar boundary to the acinar lumen (Figures 6A, 9C).

Oogenesis

Each acinus was found to contain primordial oocytes (0.56 ± 0.34 × 0.26 ± 0.06 µm), usually three to four in number and confined to the periacinar zone

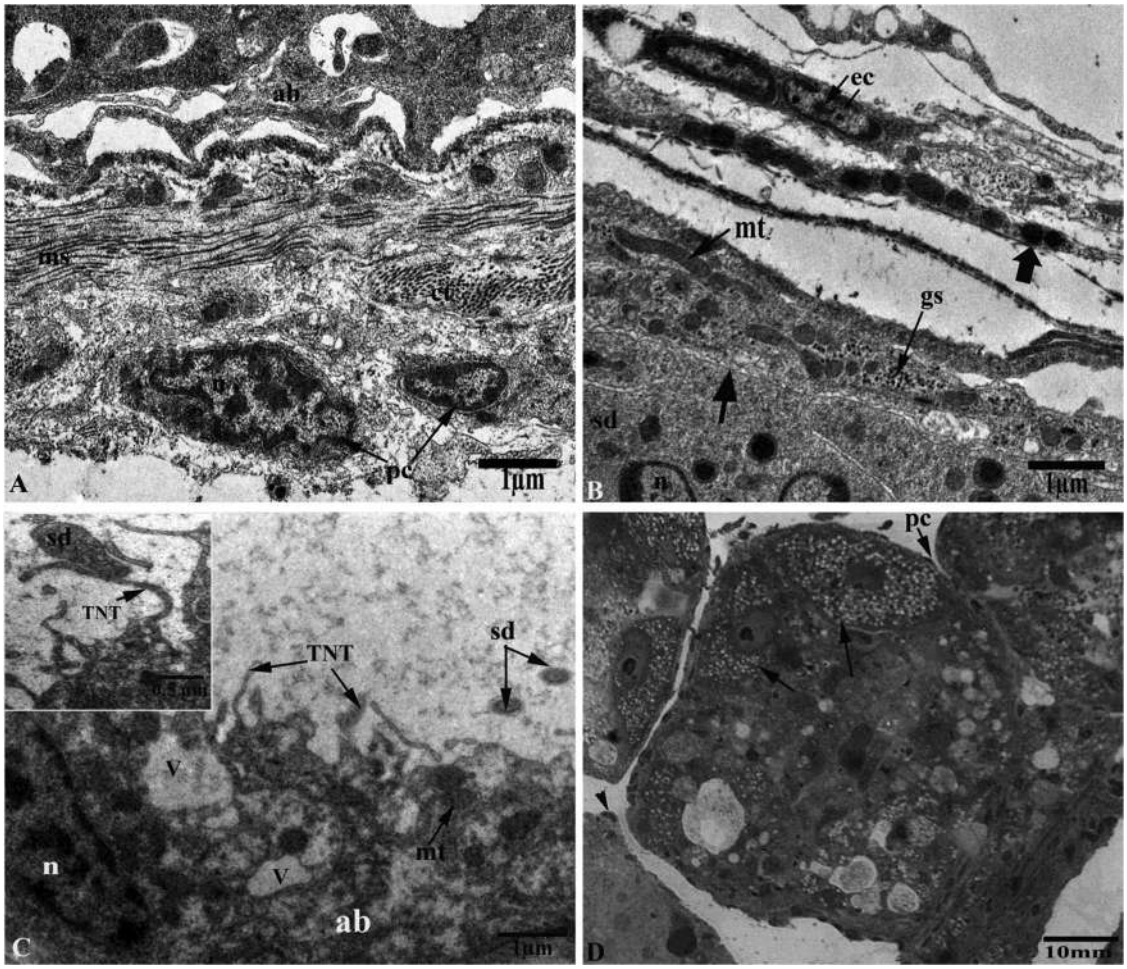

Figure 9. A–C, Transmission electron micrographs of ovotestis of smaller *Onchidium tigrinum* (0.26 ± 0.05 g). A, Junction between periacinar cell and acinar boundary; B, junction (black arrow) of developing spermatids and acinar boundary consists of secretory granules and secretory proteins (bold arrow); C, TNTs of acinar boundary. Note the connection between spermatids and TNTs inset; **D,** semi-thin section of ovotestis of larger slug showing more than one large oocyte (black arrows), arrow head indicates interacinar cell. ab—acinar boundary; ct—connective tissue; ec—elongated nucleated cell; gs—secretory granules; ms—muscle fibre; mt—mitochondria; n—nucleus; pc—periacinar cell; sd—spermatid; TNT—tunnelling nanotube; v—secretory vesicle.

(Figure 3B). One face of each oocyte was intimately associated with the acinar boundary, while the other face was directed toward the acinar lumen. Each oocyte was covered by a very thin layer of follicle cells. The cytoplasm was filled by electron opaque materials, with some lipid droplets which appeared in electron microscopy as a clear region within the intensely stained cytoplasm (Figure 3B). The nuclei of the cells were not prominent.

Larger slugs (2.38 ± 0.2 g bw)

The contents of acini were usually found to comprise two to three developed oocytes and developing spermatogenic cells (Figure 9D).

Spermatogenesis

Like smaller slugs, the acini of larger slugs contained spermatogenic cells of differing stages of development, but spermatogonial cells were absent (Figure 10A–B). In serial semi-thin sections, it was observed that the spermatogenic cells were mainly located in the central acinar region (Figures 9D, 10A).

Sertoli cells

Sertoli cells were present in the acini of larger slugs, but they were degenerate in form. The cells contained very few TNTs (Figure 10C), but the cytoplasm was rich in mitochondria, ER and secretory vesicles. The developing spermatogenic cells were not firmly attached to the Sertoli cell (Figure 10C).

Interacinar components and the acinar boundary

Regions between acini in the ovotestis of larger slugs were occupied by interacinar and periacinar cells (Figure 9D), but the number of these cells was less in the ovotestis of smaller slugs. The membranous channels of the acinar boundary were very short and mostly free of connection with the spermatogenic cells (Figures 9D, 10A–B).

Oogenesis

Each acinus possessed more than one—typically two or three—large, developed oocytes (0.82 ± 0.14 × 0.48 ± 0.12 μm) (Figures 9D, 10A) dispersed among spermatogenic cells at various stages of development (Figure 10A–D). The oocytes were typically oval in shape and

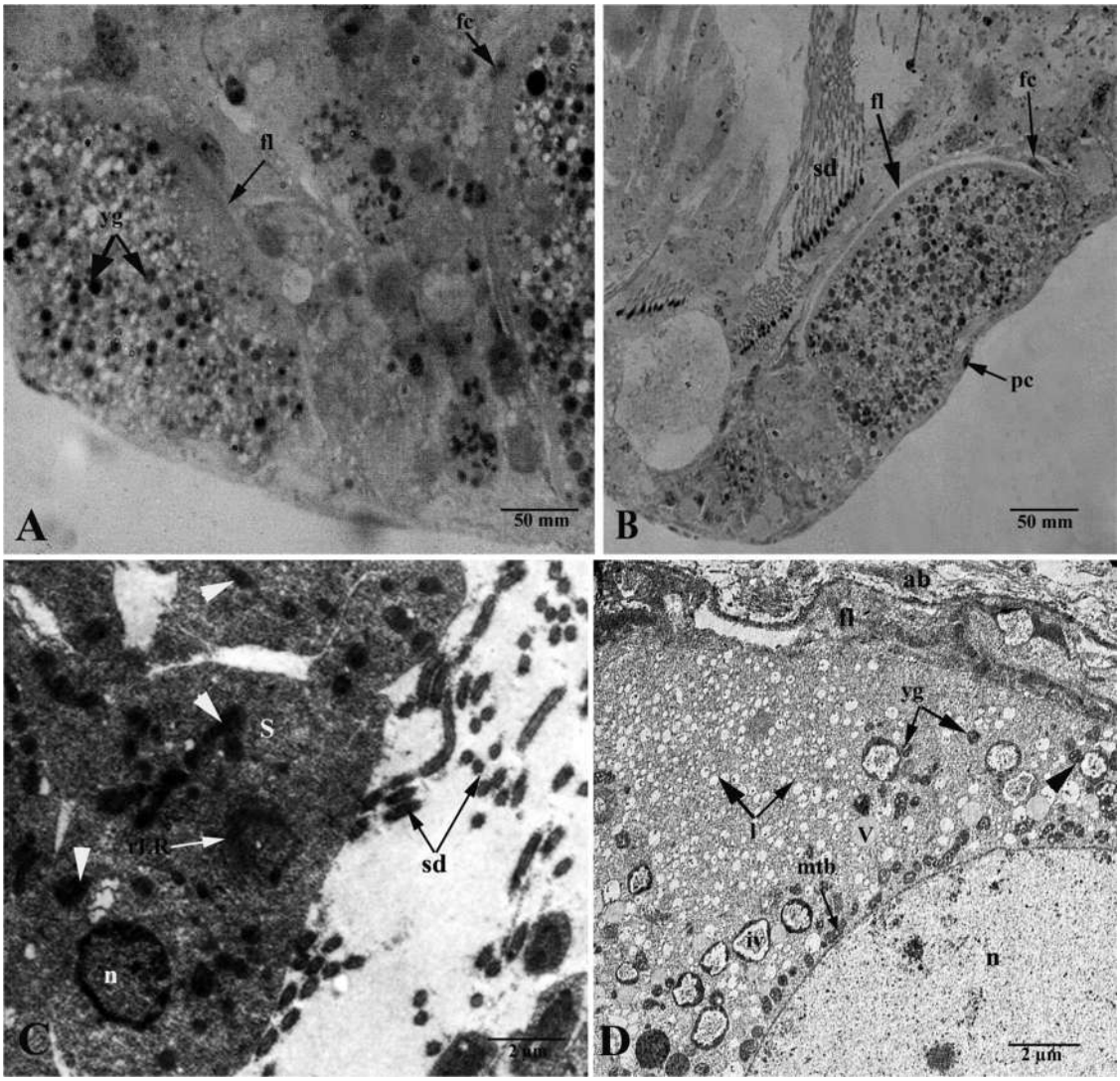

Figure 10. A–B, Semi-thin sections of the ovotestis of larger *Onchidium tigrinum* (2.38 ± 0.2 g). A, Thin follicular cell layer surrounds the oocyte; B, follicular cell layer acts as a barrier between oocyte and other acinar component; **C–D,** transmission electron microscopy of ovotestis of *Onchidium tigrinum*; C, weak connections of spermatids with Sertoli cells. An arrow head indicates a secretory vesicle; D, junction of acinar boundary and follicular cell layer. Note the accumulation (arrow head) of yolk granules. ab—acinar boundary; fc—follicular cell; fl—follicular cell layer; iv—intermediate vesicle; l—lipid droplet; mtb—mitochondrial band; n—nucleus; pc—periacinar cell; rER—rough endoplasmic reticulum; S—Sertoli cell; sd—spermatid; v—vesicle; yg—yolk granules.

surrounded by a distinct thin layer of small follicle cells. The cytoplasm of the oocytes was observed to contain numerous lipid droplets, mitochondria, ER, membrane bound vesicles, electron-opaque yolk granules of various sizes and yolk droplets. The ooplasm appeared as a mosaic matrix of electron-lucid and electron-opaque bodies (Figure 11A–B). The nuclei of these cells were large, with a prominent nucleolus (Figures 9D, 11C). In some TEM sections it was observed that the mitochondria were arranged in a single band around the nuclear membrane (Figures 10D, 11B). Serial ultrathin sections revealed that the yolk droplets production was completed through sequential intermediate developing stages (Figure 11A–B, D).

Follicle cells

The follicle cell layer comprised minute, flattened cells, each with a small elongated nucleus, and was seen to surround each oocyte so as to demarcate them from the rest of the acinar components (Figures 10A–B, 11A). The nuclei of the follicle cells occupied most of the cellular area (Figure 11A). The cytoplasm of these cells contained dense granules of various sizes.

A cross section of an ovotestis acinus from each of a smaller and larger example of *O. tigrinum* is presented schematically (Figure 12A–B) to show the comparative structural characteristics of these two body weight classes.

Discussion

The present study provides detailed histological information on the ovotestis of the estuarine hermaphrodite pulmonate mollusc *O. tigrinum*. The overall structure of the ovotestis resembles that reported for other pulmonates (Luchtel et al. 1997; Rakshit et al.

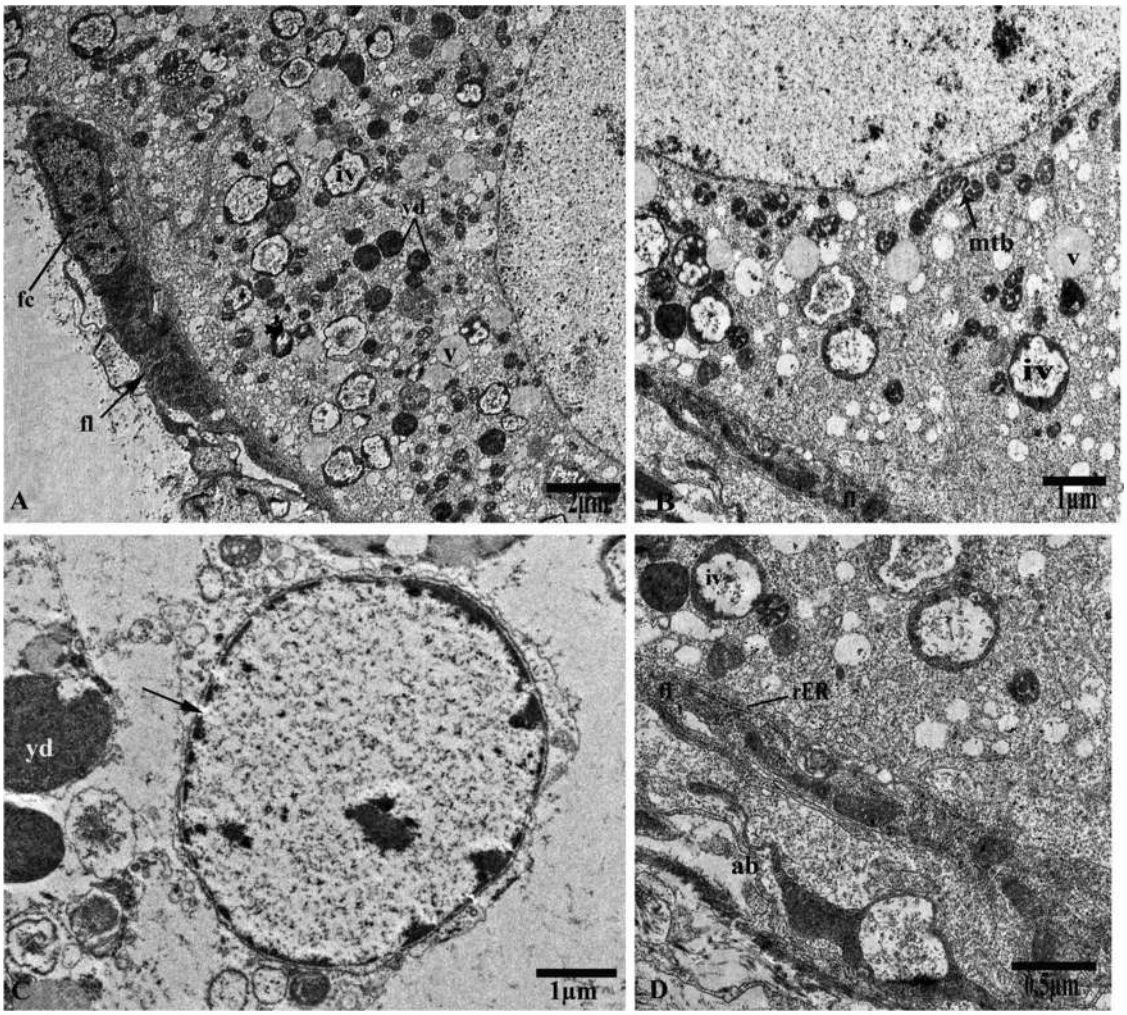

Figure 11. Ovotestis of larger *Onchidium tigrinum* (2.38 ± 0.2 g). **A–B,** Ooplasm surrounded by follicular cell layer and showing various stages of yolk droplet formation; C, oocyte nucleus with prominent nucleopore (arrow); D, magnified view of junction between acinar boundary and oocyte. ab—acinar boundary; fc—follicular cell; fl—follicular cell layer; iv—intermediate vesicle; mtb—mitochondrial band; rER—rough endoplasmic reticulum; v—vesicle; yd—yolk droplet.

2005; Roy et al. 2016). Our work included a structural comparison of the ovotestes in two body weight classes of *O. tigrinum*. Consistent with resource allocation principles (Charnov 1996) it is observed that *O. tigrinum* individuals of large body weight, presumably representing the older age class, contain well developed oocytes in their ovotestis acinus and thus can be interpreted as having the resources to invest in oogenesis. As a result, the slugs of larger body weight might act only as a sperm recipient during mating. On the other hand, small, presumably younger, slugs possess acini greatly enriched with spermatogenic cells and likely act only as potential sperm donors during copulation (cf. Ohbayashi-Hodoki et al. 2004; Jordaens et al. 2005; Hermann et al. 2009; Nakadera et al. 2015). Nonetheless, we have not specifically studied the exchange of gametes in mating pairs of *O. tigrinum*. The relationship between the numbers of oocyte per acinus and the habitat of the pulmonate clades is shown in Table 2. All Stylommatophora are terrestrial and possess one oocyte per acinus except *Archachatina marginata* (Swainson, 1821) which requires further clarification. Members of the clade Hygrophila (freshwater), marine Panpulmonata and estuarine Systellommatophora have more than one oocyte per acinus.

In the acini of smaller *O. tigrinum* the spermatogonial cells and Sertoli cells are present in large numbers (as seen in the ovotestis of other pulmonates; e.g., *Macrochlamys indica*, Roy et al. 2016). The sequential condensation of nuclear materials in the developing spermatids of *O. tigrinum* is of a similar pattern to spermiogenesis described in other pulmonates (Takaichi and Dan 1977; Takaichi 1978; Selmi et al. 1988; Pastisson and Lacorre 1996; Hodgson and Healy 1998; Rakshit et al. 2005; Mansour et al. 2011; Gamil 2013; Roy et al. 2016). However, the condensation of nuclear substances along with the nuclear membrane follows a unique pattern in each species, even varying among Onchidiidae (Bing et al. 2008; Ping et al. 2008; Chen et al. 2015).

The most important characteristics of the developing oocytes present in the acini of larger *O. tigrinum* are the formation of large yolk droplets through the

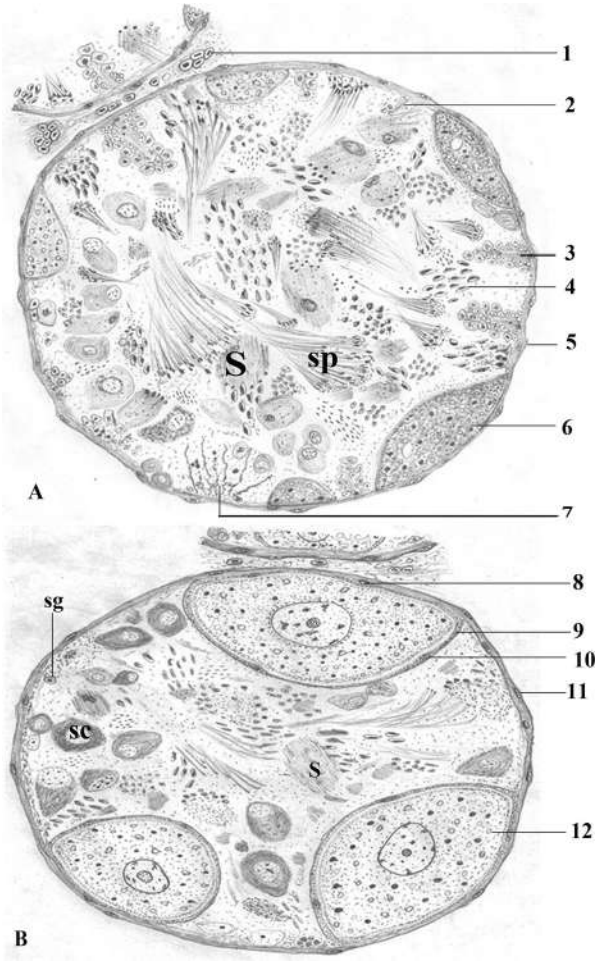

Figure 12. Diagrammatic representations of acini in ovotestes showing an acinus and its associated structures. **A,** Smaller, presumably younger *Onchidium tigrinum*; **B,** larger, presumably older *Onchidium tigrinum*. S—Sertoli cell; sc—spermatocyte; sg—spermatogonia; sp—sperm; 1—interacinar cell; 2—TNTs of acinar boundary; 3—aggregation of developing spermatogenic cells; 4—spermatids; 5—acinar boundary; 6—developing oocyte; 7—TNTs of Sertoli cell; 8—elongated nucleated cell in acinar boundary; 9—follicular cell layer; 10—follicular cell; 11—periacinar cell; 12—more developed oocytes (compared to those of smaller slugs).

accumulation of small yolk granules which conforms to other molluscs as well as some other invertebrates (Nørrevang 1968; Griffond and Bolozoni-Sungur 1986; Medina et al. 1986; Wourms 1987; Eckelbarger and Blades-Eckelbarger 1989; Eckelbarger and Davis 1996; Pal and Hodgson 2002; Amor et al. 2004; Bing et al. 2008). The developing oocytes are surrounded by a prominent, well developed follicle cell layer which clearly separates them from other acinar structures and may help in the nourishment and, probably, in the regulation of hormone signalling to the developing oocyte (O'Donovan and Abraham 1987; Pal and Hodgson 2002; Rakshit et al. 2005; Roy et al. 2016).

There is much similarity between spermatogenesis in mammals and pulmonates. Sertoli cells, interstitial cells (Leydig cells) and periacinar cells (myoid cells) are present in both of these widely separated animal taxa and their functions are likely to be similar. The existence of TNTs in Sertoli cells and other cells is now added to these similarities. This study describes for the first time the detailed structural characteristics of Sertoli cells, interacinar cells, periacinar cells and the acinar boundary in the ovotestis of *Onchidium tigrinum*. In the mammalian system, it is known that TNTs create a membranous bridge between cells and between tissue and cells, and support the supply of various small cellular components and secretory materials (Guo and Zheng 2004; Gerdes et al. 2007; Jiang Hui and YouYi 2013). In *O. tigrinum* the TNTs are apparently mainly necessary for the development of spatially-distantly gametes as they are extensively present in the ovotestis of smaller slugs where there is a predominance of spermatogenesis and developing spermatogenic cells are distributed throughout the acinar space. In the ovotestis of larger slugs, TNTs are less abundant due to predominance of oogenesis that is restricted in the periphery of acini. Earlier, Roy et al. (2016) studied ovotestis of *Macrochlamys indica* and documented the presence of thin membranous cellular channels communicating between cells of the acinar boundary and Sertoli cells (cf. Figure 5A, arrow). We now consider that these thin membranous channels in *M. indica* are better interpreted as TNTs. The TNTs in the ovotestis of *O. tigrinum* are here recognised for the first time in the ovotestis of molluscs. It is presumed that the periacinar cells may be functionally similar to the peritubular myoid cells in the mammalian seminiferous tubules and promote passage of the mature sperm into the hermaphroditic duct through the acinar lumen by their peristaltic waves (Skinner and Fritz 1985; Maekawa et al. 1996; Roy et al. 2016).

In the acini of *O. tigrinum*, the peripheral area shows some prominent connections between the acinar boundary, developing gametes and Sertoli cells. The acinar boundary and Sertoli cells maintain an intimate association with the cells of gametogenetic cells, either directly or by thin membranous channels (TNTs). These connections may be responsible for supplying nutritional requirements for the development of gametes (Johnson et al. 2008). Such connections between acinar boundary and gametogenic cells may regulate the hormonal supply in the developing gametes which, in an earlier study, was shown to depend on the age of the animals (Roy et al. 2016). The gradual enhancement of VSI along with the increase in body weight may be the result of acumulation of vitellogenin/ferritin in the albumen gland. The gradual decrease of VSI in the older slugs (1.09–3.57 g) might be due to the expenditure of the vitellogenin/ferritin of the albumen gland for oogenesis in the acini. It may be assumed that low VSI is indicative of functional spermatogenesis and that higher values of VSI are suggestive of proliferative oogenesis.

Table 2. Comparative data of the number of oocytes per acinus in various different pulmonate molluscs living in different habitats.

Clades	Species name	Habitat	Oocytes/acinus	References
Stylommatophora	*Achatina fulica*	Terrestrial	1	Rakshit et al. (2005)
Stylommatophora	*Macrochlamys indica*	Terrestrial	1	Roy et al. (2016)
Stylommatophora	*Helix aspersa*	Terrestrial	1	Griffond and Bolozoni-Sungur (1986)
Stylommatophora	*Archachatina marginata*	Terrestrial	1 or 2 (rare)	Odiete (1982)
Hygrophila	*Biomphalaria glabrata*	Freshwater	> 2	De Jong-Brink et al. (1976)
Hygrophila	*Lymnaea stagnalis*	Freshwater	> 2	De Jong-Brink et al. (1981)
Panpulmonata	*Siphonaria capensis*	Marine	> 2	Pal and Hodgson (2002)
Panpulmonata	*Siphonaria serrata*	Marine	> 2	Pal and Hodgson (2002)
Systellommatophora	*Onchidium reevesii* (*O. struma*)	Estuarine	> 2	Bing et al. (2008)
Systellommatophora	*Onchidium tigrinum*	Estuarine	> 2	Present study

The interacinar spaces of *O. tigrinum* are occupied by connective tissue, blood vessels and aggregations of small oval cells that have previously been considered as interstitial cells (Rakshit et al. 2005), but may be termed 'interacinar cells' because of their location between the acini of the ovotestis. These cells are functionally similar to those of interstitial cells (Leydig cells) found in the seminiferous tubules of the mammalian testis (Omran 2012). In the ovotestis of young *O. tigrinum* the interacinar cells are likely to produce male specific hormones and help in spermatogenesis.

Gametogenesis in the ovotestis of *O. tigrinum* is directly correlated with weight (and size) variation. Hence, the relationship between types and nature of gametogenesis and the body weight of *O. tigrinum* predetermines the functional role to be assumed in copulation (Otsuka et al. 1980). The significant development and distribution pattern of somatic cells, mainly the interacinar cells, periacinar cells and Sertoli cells, indicate a correlation with the abundance of the spermatogenic cells in the ovotestis (Parivar 1980; Maekawa et al. 1996; Pastisson and Lacorre 1996).

The overall structural features of the ovotestis of *O. tigrinum* are more similar to those of aquatic hermaphrodite pulmonates (De Jong-Brink et al. 1981; Bergmann et al. 1984; Pal and Hodgson 2002) than terrestrial stylommatophorans (Rakshit et al. 2005; Roy et al. 2016). The diversity of oocyte production in the ovotestes of hermaphrodite pulmonate species (Table 2) may have significance in adaptation and maximisation of reproductive fitness in the respective habitats of the species (e.g., Bateman 1948; Angeloni and Bradbury 1999), but may also be, at least partly, phylogenetically determined.

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No potential conflict of interest was reported by the authors.

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